# LOCALIZATION OF ACTIN IN *DICTYOSTELIUM* AMEBAS BY IMMUNOFLUORESCENCE

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ABSTRACT

Antibody prepared against avian smooth muscle actin has been used to localize actin in the slime mold, *Dictyostelium discoideum*. The distribution of actin in migrating cells is different from that in feeding cells. Migrating amebas display fluorescence primarily in advancing regions whereas feeding amebas show uniform fluorescence throughout. The reaction is specific for actin since the fluorescence observed is blocked when the antibody is absorbed by actin purified from avian skeletal muscle, human platelets, and *Dictyostelium*. These results, in addition to describing the distribution of actin in *D. discoideum*, demonstrate that actins from these diverse sources share at least one common antigenic determinant.

KEY WORDS cellular slime mold · cell motility · antibody against actin · microfilaments · cell morphology

Directed cell motility is an integral part of the life cycle of the cellular slime mold, *Dictyostelium discoideum*. This life cycle is divided into stages, each having different activities associated with cell motility. During the feeding stage, cell division and phagocytosis are major activities of the cells (8). Later, during the aggregation phase, migratory activity becomes important and the rate of motility increases (19).

The relationship between these activities and quantitative changes in actin has been demonstrated (5), but the distribution of actin during different life cycle stages has not become clear. A cortical layer of actin has been demonstrated by heavy meromyosin binding (3, 5). However, regional differences in the actin layer cannot be observed because of the disruptive nature of this technique.

In the present paper, we have used antibodies specific for actin (11, 13) to examine actin distribution in *Dictyostelium* amebas. This procedure has allowed visualization of regional differences in actin distribution which correlate with changes in cellular activity. In addition, we demonstrate by absorption analysis that actins from *D. discoideum*, avian smooth muscle, and human platelets share at least one antigenic determinant.

## MATERIALS AND METHODS

### *Scanning Electron Microscopy*

Amebas of *Dictyostelium* were fixed in 3% glutaraldehyde, 1% paraformaldehyde buffered with 0.05 M sodium cacodylate, pH 7.2. The cells were dehydrated in acetone and critical-point dried for scanning microscopy according to Porter et al. (18).

## Cell Culture

*D. discoideum* strain NC-4 (haploid) was grown on lactose agar (0.1% peptone, 2% agar) with *Escherichia coli* strain B/r as a food supply. Cells were washed from the agar with Bonner's (1) saline and allowed to settle on Formvar films coated with polylysine (3). Some Formvar films were treated for 5 min with 20 mg/ml 1-cyclohexyl-3-(2-morpholinoethyl-carbodiimide metho-*p*-toluene sulfonate) (Sigma Chemical Co., St. Louis, Mo.), and then for 5 min with 2 mg/ml protamine sulfate followed by 1 h in a 1:1 mixture of each (G. Veomett, personal communication). This surface was slightly better than polylysine in providing firm attachment for the cells.

## Indirect Immunofluorescence

Antibodies to chicken gizzard actin were prepared according to methods described elsewhere (11, 12). Cells were examined to determine their pattern of movement, and selected positions were noted for later relocation. Cells were fixed under observation for 20 min in 3.7% formaldehyde in phosphate-buffered saline (PBS). After a brief rinse in PBS, the cells were placed in 95% ethanol for 10 min. Antibody for actin was applied to the cells after rehydration in PBS, and the preparations were incubated for 1 h at 37°C. The cells were rinsed with PBS and treated with fluorescein-labeled goat anti-rabbit IgG (Miles Laboratories Inc., Elkhart, Ind.) for 1 h at 37°C. Observations were made on a Leitz Orthoplan microscope equipped with a mercury arc lamp and epifluorescence optics. Micrographs were taken on Kodak Plus-X film; the film was developed on Diafine (Acufine Inc., Chicago, Ill.).

## Purification of Dictyostelium Actin

*Dictyostelium* cells were harvested from liquid culture (22) and washed several times with Bonner's (1) saline. Pelleted cells were resuspended in an equal volume of 10 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$, and homogenized with a Dounce homogenizer (Kontes Co., Vineland, N. J.). After standing for 20 min at 4°C, the homogenate was centrifuged for 5 min at 1,000 *g*. Actin was purified from the supernate by affinity chromatography on a DNase-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column (14). Fractions containing *Dictyostelium* actin (the 3-M guanidine HCl eluate) were pooled and dialyzed against 10 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$ for use to absorb the chicken gizzard actin antibody.

## Absorption of Antibody

Actins purified from skeletal muscle (21), human platelets (17), and from *Dictyostelium* (by the above described method) were denatured by heating to 90°C for 10 min. Platelet actin was further purified before denaturation by precipitation with ammonium sulfate at 30% of saturation. 100–150 μg of denatured actin was mixed with 100 μl of antibody (0.20 mg/ml). The purity of the antigens used for absorption is shown in Figs. 8–10. After standing at 4°C overnight, the precipitate was removed by centrifugation and the supernate was used for indirect immunofluorescence as described above. Using this technique, all actin-specific antibody, including monovalent forms, should be removed in the precipitate.

Native actin from chicken gizzard was used to absorb antibody by affinity chromatography. A 6 x 45-cm column of DNase-Sepharose 4B (14), containing ~300 μg of DNase I, was loaded with purified chicken gizzard actin (4) by passing through an excess of the protein. 500 μl of antibody preparation (0.20 μg/ml of protein in PBS) was then passed through the column to find out whether the antibody specific for actin would be retained by the actin-DNase complex bound to the resin. The solution that passed through was lyophilized, redissolved in 500 μl of distilled water, and tested on cells by immunofluorescence.

## Electrophoresis

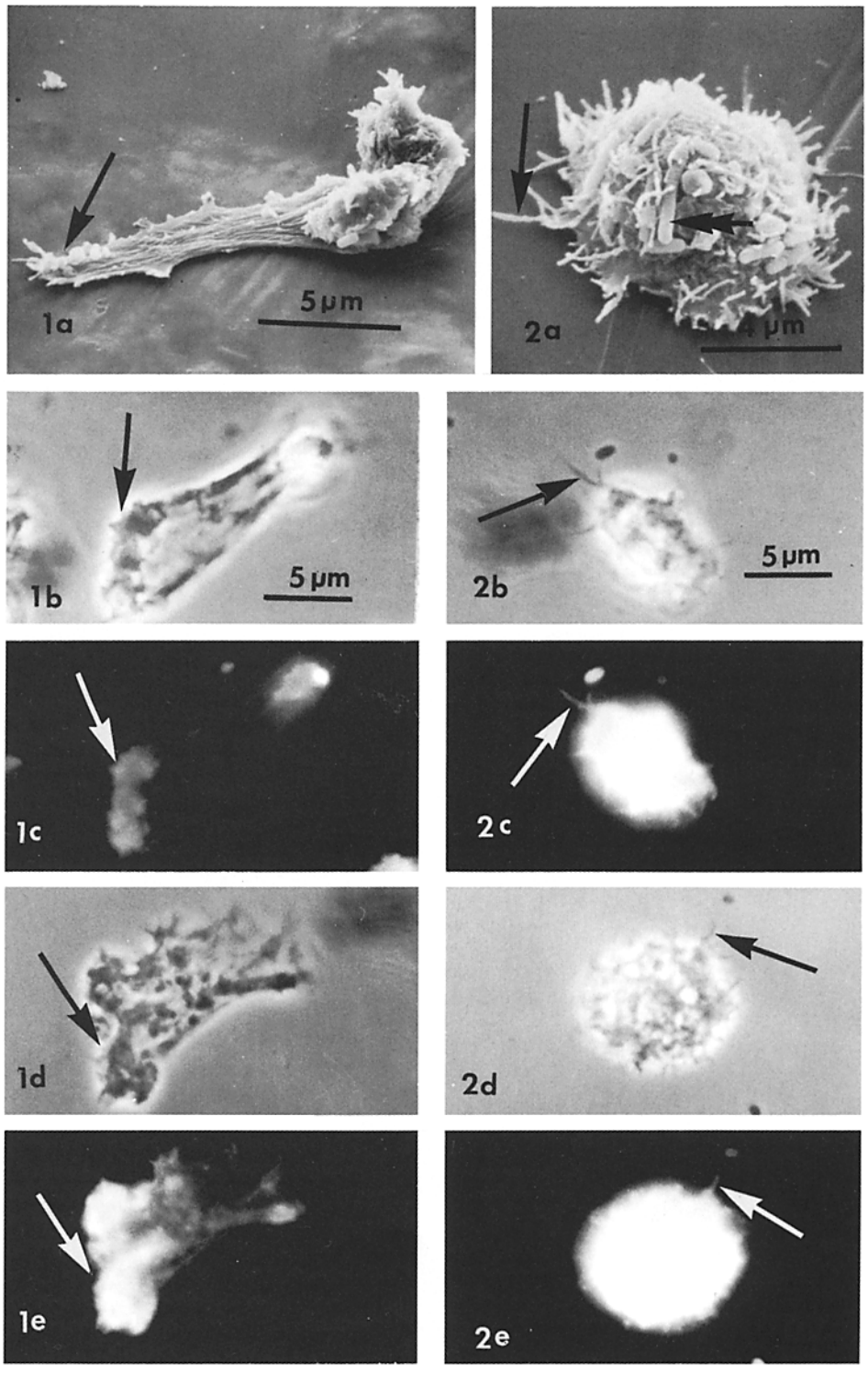
The purity of the antigens was assayed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (12.5% in acrylamide concentration) using the method of Laemmli (10) modified for slab gels.

## Immunoelectrophoresis

Agarose (Sigma Chemical Co.) plates were prepared on microscope slides using 1% agarose in sodium pyrophosphate buffer, pH 9.0 (6). Electrophoresis of the antigens was carried out for 1 h at 15 mA per plate. Sodium pyrophosphate also served as the tank buffer. After electrophoresis, antibody was applied to troughs in the gel and the plates were incubated for 36 h at 37°C. The plates were then washed for 24 h in PBS, stained with Coomassie Brilliant Blue R (ICI United States, Inc., Wilmington, Del.), and photographed.

# RESULTS

In migrating *D. discoideum* amebas of the aggregation stage, actin is found primarily in the advancing regions of the cell. The advancing areas were identified in the phase-contrast microscope just before fixation of the cells. The leading edge shows bright fluorescence after reaction with antibody specific to actin (Fig. 1*c* and *e*). Filopodia and pseudopodia are also brightly fluorescent. Regions of the cytoplasm behind the leading edge are virtually unreacted, as is the tail of many cells. The bright fluorescence at the leading edge is not accompanied by an increase in thickness. This is particularly well demonstrated in scanning electron micrographs of cells comparable to those



studied by immunofluorescence (Fig. 1*a*). In some cells (Fig. 1*b*), a large body of cytoplasm, much thicker than the rest of the cell, may be found in the tail. Fluorescence in this thicker area indicates the presence of some actin in this region (Fig. 1*c*). Faint fluorescence is often detected along the sides of migrating cells. In all cases, no substructure or other indication of organization within the actin-containing regions is detected.

Feeding amebas show a different distribution of actin as well as a different morphology. Feeding cells are round with many filopodia on the entire surface (Fig. 2*a*, *b*, and *d*). Treatment of these cells with antibody against actin shows no polarization of actin distribution (Fig. 2*c* and *e*). The actin appears to be distributed throughout feeding cells. Filopodia, when visible, also show fluorescence (Fig. 2*c* and *e*).

The specificity of the chick smooth muscle actin antibody is demonstrated by absorption with purified actin. When the antibody preparation is absorbed with actin purified from chick skeletal muscle (Figs. 3 and 8), human platelets (Figs. 4 and 9), or *Dictyostelium* (Figs. 5 and 10), the fluorescent reaction is not observed. Attempts to absorb antibody specific for chick gizzard actin with other heat-denatured proteins such as tubulin or collagen were unsuccessful. Similarly, saturation of DNase I bound covalently to Sepharose 4B with purified native chick gizzard actin and subsequent passage of the antibody to chick gizzard actin through such an affinity column also removes the ability of the effluent globulin fraction to react with cells in indirect immunofluorescence (Fig. 7). Passage of the antibody through a column of DNase I-Sepharose 4B which does not contain purified chick gizzard actin does not result in absorption of the antibody. The fluorescence, however, is not entirely removed from the bacteria when present in the samples. No specific fluorescence pattern is observed with either preimmune serum (Fig. 6) or fluorescein-conjugated goat anti-rabbit IgG alone (result not shown), although bacteria in these samples continue to show fluorescence.

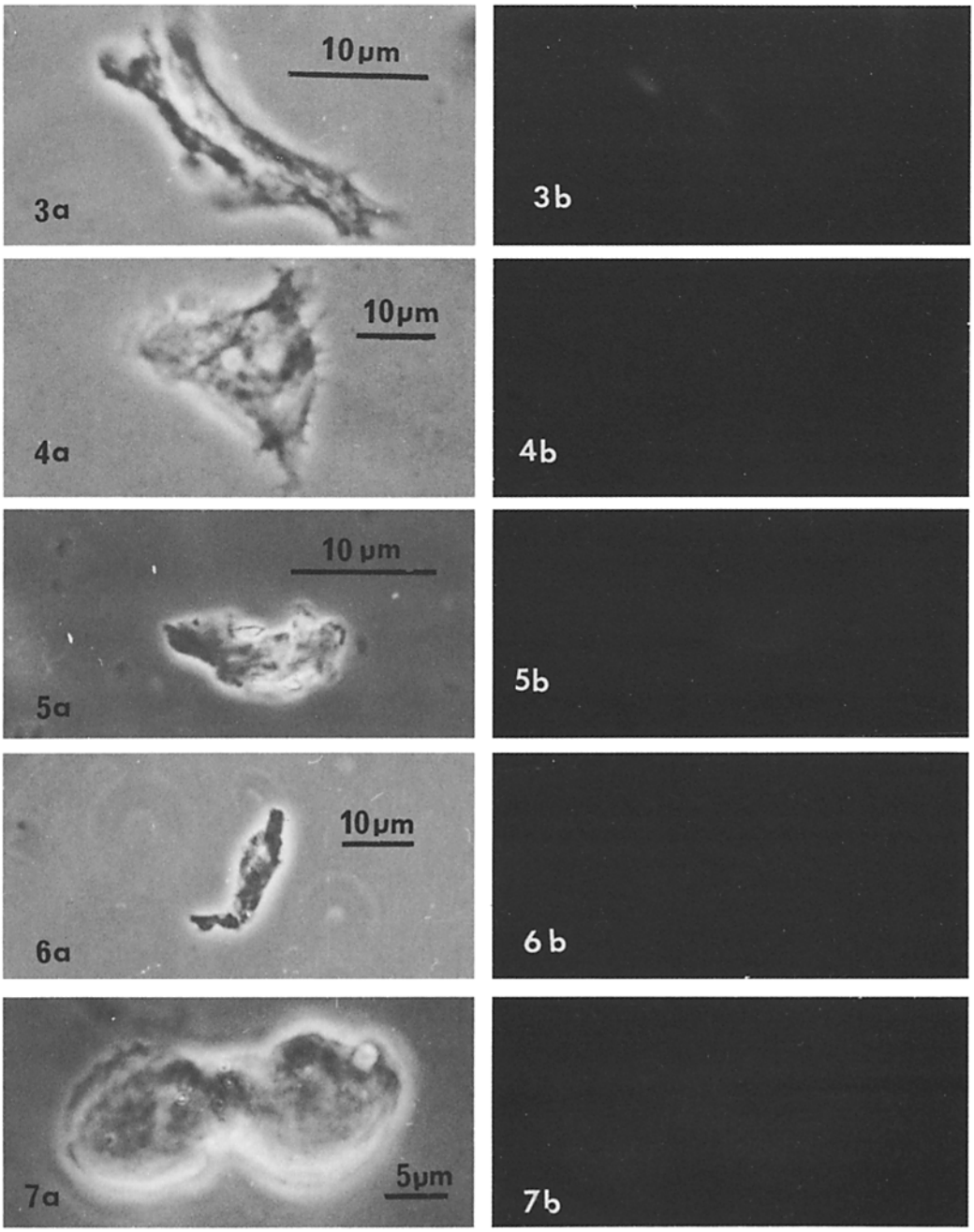
Immunoelectrophoresis results in one precipitin band when either purified platelet actin or chicken gizzard actomyosin is used as antigen (Fig. 12). A slightly longer band is formed on the actomyosin side of Fig. 12.

## DISCUSSION

The filamentous appearance of *Dictyostelium* actin in the electron microscope is well known. Previous observations of *Dictyostelium* amebas have demonstrated a cortical layer containing actin filaments which bind heavy meromyosin (3, 5). However, after conventional glutaraldehyde and osmium fixation for electron microscopy, only a few filaments are observed (5). The immunofluorescence data presented here establish that actin is present in the cell cortex without glycerination. It has recently been shown that the absence of actin-binding proteins such as the tropomyosin leads to destruction of filaments by osmium fixation (23). The absence of numerous filaments from the cortical layer of *Dictyostelium* amebas may result from such disruption by osmium.

FIGURE 1 (*a*) Scanning electron micrograph of a migrating *Dictyostelium* amoeba. The leading edge is indicated by the arrow. Note the large body of cytoplasm in the tail of the cell. × 4,000. (*b*) Phase-contrast micrograph of a migrating cell comparable to that in Fig. 1*a*. The leading edge is indicated by the arrow. Note a large, out of focus, cytoplasmic mass in the tail. × 2,500. (*c*) Same cell as Fig. 1*b* observed by epifluorescence optics. Note the intense fluorescence in the leading edge of the cell (arrow) resulting from reaction with antibody against avian smooth muscle actin. Fluorescence is also visible in the thick tail of the cell. × 2,500. (*d*) Phase-contrast micrograph of a migrating amoeba. The tail lacks the large cytoplasmic mass. The leading edge is indicated by the arrow. × 2,500. (*e*) The same cell as Fig. 1*d* observed by epifluorescence. Binding of antibody specific for actin results in fluorescence in the leading edge. × 2,500.

FIGURE 2 (*a*) Scanning electron micrograph of a feeding amoeba of *Dictyostelium*. Note numerous filopodia (arrows) and captured *E. coli* (double-headed arrow). × 5,000. (*b*) Phase-contrast micrograph of a cell comparable to that in Fig. 2*a*. Note filopodium (arrow). × 2,500. (*c*) Epifluorescence micrograph of the same cell as Fig. 2*a*. Reaction with antibody against actin results in fluorescence throughout the cell. Note filopodium (arrow). × 2,500. (*d*) Phase-contrast micrograph of a cell similar to that in Fig. 2*b*. Note filopodium (arrow). × 2,500. (*e*) Epifluorescence micrograph of the cell shown in Fig. 2*d*. Fluorescence results from reaction with antibody against actin. Note filopodium (arrow). × 2,500.




FIGURES 3-7 Immunofluorescence controls of *Dictyostelium* amebas. (3*a*, 4*a*, 5*a*, 6*a*, and 7*a*) Phase-contrast micrographs of *Dictyostelium* amebas. (3*b*, 4*b*, 5*b*, 6*b*, and 7*b*) *Dictyostelium* amebas observed by epifluorescence microscopy. (3*b*) The same cell as Fig. 3*a*. The antibody was absorbed by chick skeletal muscle actin (see Fig. 8), removing the fluorescence of the specific reaction. Faint fluorescence is visible in an ingested *E. coli*. × 2,000. (4*b*) The same cell as Fig. 4*a*. The antibody has been absorbed by human platelet actin (see Fig. 9). × 2,000. (5*b*) The same cell as Fig. 5*a*. The antibody has been absorbed by actin purified from *Dictyostelium* (see Fig. 10). × 2,000. (6*b*) Same cell as Fig. 6*a*. The cell was treated with rabbit preimmune serum and prepared for immunofluorescence. × 1,000. (7*b*) Same cell as Fig. 7*a* observed by epifluorescence microscopy. The antibody was absorbed by native chick gizzard actin bound to DNase-Sepharose 4B. × 2,000.

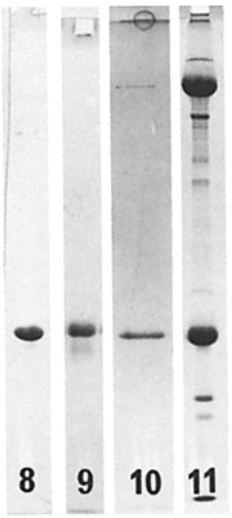



FIGURE 8 Polyacrylamide gel electrophoresis of chick skeletal muscle actin purified according to Spudich and Watt (21). This sample was used to absorb antibody (see Fig. 3).

FIGURE 9 Polyacrylamide gel electrophoresis of a 0–30% ammonium sulfate cut of human platelet actin purified according to Pollard et al. (17). This sample was used to absorb antibody (see Fig. 4).

FIGURE 10 Polyacrylamide gel electrophoresis of the 3-M guanidine eluate from a DNase-Sepharose 4B column which had been loaded with the low salt extract of *Dictyostelium* amoebae. Note presence of a high molecular weight protein.

FIGURE 11 Polyacrylamide gel electrophoresis of chicken gizzard actomyosin prepared according to Driska and Hartshorne (4). This sample was used for immunoelectrophoresis (see Fig. 12).

Changes in distribution of cortical actin are suggested by the different patterns of fluorescence in feeding and migrating cells. A local increase in the amount of actin at the leading edge of migrating cells is suspected as the cause of increased fluoresence in this area. This suggests that the distribution of actin in the cells may be related to the sites of activity where this protein is required. If this is so, the feeding cell, with its more uniform distribution of actin, may be potentially active over its entire surface. This may be essential for immediate phagocytic response to contact with bacteria.

Changes in the distribution of actin suggested by these observations may require changes in the form of actin within the cell. Spudich and Cooke (20) studied forms of *Dictyostelium* actin and found that it will form paracrystals in the presence of magnesium and nets in the presence of calcium. *Dictyostelium* actin will also form 6-nm filaments like those of skeletal muscle actin. Furthermore, the formation of a gelled state by actin in cytoplasmic extracts of *Acanthamoeba* cells has been demonstrated (16). Recent work has shown that such gelled states of actin and associated proteins exist under physiological conditions and may represent a real functional state of these proteins within the cell (24). Distribution changes of actin, suggested by our observations, may require transitions through one or more of these actin forms.

Directional changes of *Dictyostelium* amebas induced by local applications of cyclic AMP occur within 5 s (7). Many of these micropipette applications of cyclic AMP were at regions of the cell with very little antibody labeling. This suggests the possibility of rapid redistribution of actin from the old leading edge to the new advancing area.

Our immunofluorescence is specific for actin. This is indicated by the fact that the antibody raised against avian smooth muscle actin, purified by SDS polyacrylamide gel electrophoresis before immunization, is absorbed by native or heat-denatured heterologous actins prepared by other biochemical methods and not absorbed by other heat-denatured proteins (2). Absorption by DNase-purified *Dictyostelium* actin provides particularly strong evidence for the specific labeling

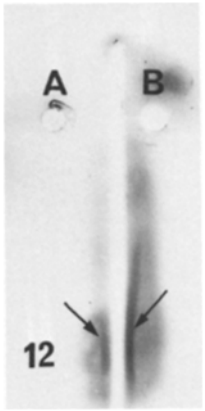



FIGURE 12 Immunoelectrophoresis of antibody specific for actin. Well A was filled with purified platelet actin (Fig. 9) and well B was filled with chicken gizzard actomyosin (Fig. 11). After electrophoresis, the trough was filled with antiserum. A single precipitin band is visible on each side of the trough (arrows). Background staining is also visible due to Coomassie Blue R staining.

of actin in the slime mold amebas and for the usefulness of the antibody in studying the localization of actin in lower eukaryotic cells. These observations, plus that of absorption of antibody by actin-DNase complex, demonstrate specific reaction of the antibody with native or denatured actin.

Fluorescence in bacteria is apparently due to nonspecific staining. Failure of purified actin to absorb the stain from the bacteria, as well as the observation of fluorescence in bacteria after treatment with preimmune serum, suggests nonspecific staining. In addition, bacteria also show fluorescence after treatment with fluorescein-conjugated goat anti-rabbit IgG alone. The level of nonspecific staining appears to be much higher in bacteria than in slime mold cells. Because of this, cells that are rounded (e.g. the rounded nonfeeding cell in Fig. 7) do not show high levels of nonspecific fluorescence, despite their increased thickness.

The uniform fluorescence of feeding cells is apparently not due to coating of the cells by bacteria which fluoresce nonspecifically. Scanning electron micrographs of feeding cells (Fig. 2*a*) show numerous bacteria on the surface of the cells. These bacteria, however, do not form a uniform layer. If these bacteria were the only source of fluorescence, a mottled pattern would appear. The fluorescence of bacteria is, in fact, masked by the bright fluorescence of the antibody staining of actin. In addition, the filopodia (Fig. 2*c* and *d*) of these cells are considerably smaller than bacteria and would not show fluorescence unless antibody staining of actin were occurring within these structures.

A further concern over specificity is raised by the recent report of electrophoretic co-migration of actinin and actin in *Physarum* (9). Our absorption controls indicate that no antibody against actinin contributes to the specific staining pattern since this pattern is absent in these controls. The protein samples used were prepared in such a way that actinin should not be present and therefore antibody against actinin would not be absorbed. Platelet actin purification included a salting-out step at 30% of saturation which would leave actinin in solution (9). Furthermore, actinin should not be present in the DNase-actin column used for absorption.

This study shows that actins from different contractile systems must share at least one common antigenic determinant group. The presence of this common determinant results in the ability of antibodies made against SDS-denatured chicken gizzard actin to cross-react with actins from other sources. Owaribe and Hatano (15) have shown that antibodies made against native or SDS-denatured actin from *Physarum* will not cross-react strongly with actin from skeletal muscle. This suggests that *Physarum* actin possesses determinant sites different from those of skeletal muscle actin. Thus, while at least some of the determinants of higher eukaryotic muscle and nonmuscle actin may be present on actin from lower eukaryotic nonmuscle cells, the latter actins may possess unique determinants which set them apart from higher eukaryotic action.

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